DOI: 10.1016/j.foodchem.2013.08.097

Susceptibility of milk protein-derived peptides to dipeptidyl peptidase IV (DPP-IV) hydrolysis

Alice B. Nongonierma & Richard J. FitzGerald*

Department of Life Sciences and Food for Health Ireland (FHI), University of Limerick, Limerick, Ireland.

Please cite as:
Nongonierma, A. B. & FitzGerald, R. J. (2014). Susceptibility of milk protein-derived peptides to dipeptidyl peptidase IV (DPP-IV) hydrolysis. Food Chemistry, 145, 845-852.

*Corresponding author: dick.fitzgerald@ul.ie
Tel: +353 (0) 61 202598
Fax: + 353 (0) 61 331490

Abstract

In silico digestion of milk protein-derived peptides with gastrointestinal enzyme activities was used to predict the release of peptides with a Pro residue at position 2 from the N terminus. These peptides are known to act as preferred dipeptidyl peptidase IV (DPP-IV) substrates. Five casein-derived synthetic peptides (Ile-Pro-Ile-Gln-Tyr, Leu-Pro-Leu-Pro-Leu, Tyr-Pro-Tyr-Tyr, Leu-Pro-Tyr-Pro-Tyr and Ile-Pro-Ile) and a casein (CasH), whey (WPH) and lactoferrin hydrolysate (LFH) generated with gastrointestinal enzymes were incubated with DPP-IV at 37°C for 18 or 24 h. Peptide breakdown was evident following incubation with DPP-IV. Different modes of DPP-IV inhibition were observed depending on the test compound. Ile-Pro-Ile-Gln-Tyr, Tyr-Pro-Tyr-Tyr and Leu-Pro-Tyr-Pro-Tyr were substrate-, Leu-Pro-Leu-Pro-Leu and CasH were prodrug-while WPH and LFH were true DPP-IV inhibitors. These results are relevant for the bioactivity and bioavailability of functional foods targeting DPP-IV inhibition with potential blood glucose regulatory properties in humans.

Key words: dipeptidyl peptidase IV inhibitors, substrate-type inhibition, prodrug-type inhibition, antioxidant, bioactive peptides, milk

1. Introduction

Inhibition of dipeptidyl peptidase IV (DPP-IV) has been proposed as a new avenue for the treatment of Type 2 diabetes (T2D). DPP-IV is an ubiquitous enzyme which can be found in different locations of the body including the surface of various cells and in the circulation. It hydrolyses incretin hormones such as glucose dependent insulinotropic peptide (GIP) and glucagon-like polypeptide-1 (GLP-1). Those incretins can enhance insulin secretion from pancreatic beta cells in the presence of nutrients i*n vivo* (Bjelke et al., 2006). The degradation of GLP-1 and GIP by DPP-IV results in a loss in the bioactive properties of these hormones. DPP-IV drug inhibitors are utilized to prevent incretin degradation *in vivo*, thereby increasing their half-life (Bjelke et al., 2006).

Various studies have highlighted the possibility of using food-derived proteins and peptides as a natural source of DPP-IV inhibitors (Hatanaka et al., 2012; Huang, Jao, Ho & Hsu, 2012; Lacroix & Li-Chan, 2012b). These sources notably include milk proteins (Lacroix & Li-Chan, 2012a; Silveira, Martínez-Maqueda, Recio & Hernández-Ledesma, 2013; Tulipano, Sibilia, Caroli & Cocchi, 2011; Uchida, Ohshiba & Mogami, 2011; Uenishi, Kabuki, Seto, Serizawa & Nakajima, 2012). Casein and whey protein hydrolysates have been identified as DPP-IV inhibitors (Lacroix & Li-Chan, 2012a; Nongonierma & FitzGerald, 2013; Tulipano et al., 2011; Uenishi et al., 2012). Hydrolysates generated from β-lactoglobulin (Lacroix & Li-Chan, 2013; Silveira et al., 2013; Uchida et al., 2011), α-lactalbumin (Lacroix & Li-Chan, 2013), bovine serum albumin (Lacroix & Li-Chan, 2013) and lactoferrin (Lacroix & Li-Chan, 2013; Nongonierma & FitzGerald, 2013) have also been identified as potent DPP-IV inhibitors. Various DPP-IV inhibitory peptide sequences have been reported in the literature (Lacroix & Li-Chan, 2012b). The potent DPP-IV inhibitory peptides, diprotin A (Ile-Pro-Ile) and B (Val-Pro-Leu), originating from microbial cultures of *Bacillus cereus*, display half maximal inhibitory concentration (IC_{50}) values in the μM range (Rahfeld, Schierborn, Hartrodt, Neubert & Heins, 1991). Recently, different dipeptides from rice protein with a Pro residue at the C terminus have

been identified as DPP-IV inhibitors (Hatanaka et al., 2012). However, various dipeptide sequences without Pro residues have also been identified as potent DPP-IV inhibitors (Nongonierma & FitzGerald, 2013). Several food-derived DPP-IV inhibitory peptide sequences longer than 2 amino acid residues have also been reported in recent studies (Silveira et al., 2013; Tulipano et al., 2011; Uchida et al., 2011; Uenishi et al., 2012).

It is recognised that peptides which act as inhibitors of key enzymes in metabolic pathways may have different susceptibilities to further cleavage on binding to these enzymes. For instance, it has been proposed that inhibition of angiotensin I converting enzyme (ACE), a key enzyme in blood pressure control, by peptide inhibitors could be classified into three main categories based on their stability toward ACE. Peptides displaying an inhibitor-type behaviour are not cleaved following incubation with ACE. Peptides with a prodrug-type behaviour are cleaved by ACE, resulting in the release of a "true" inhibitory peptide with a lower IC_{50} value compared to the parent peptide. The third category of peptides, which show a substrate-type behaviour, are cleaved following incubation with ACE, resulting in an increase in the IC_{50} value compared to the parent peptide (Fujita & Yoshikawa, 1999). It has been demonstrated that many DPP-IV inhibitory peptides behaved as substrates for this enzyme. Rahfeld et al. (1991) reported for the first time that diprotin A and B were substrates for DPP-IV. This result could have been anticipated as both peptides have the structural features of DPP-IV preferred substrates, where a Pro residue is located at the penultimate position (Kühn-Wache, Bär, Hoffmann, Wolf, Rahfeld & Demuth, 2011; Vanhoof, Goossens, De Meester, Hendriks & Scharpé, 1995). Milk proteins and particularly caseins are relatively rich in Pro residues, therefore, it could be anticipated that various peptide sequences showing structural characteristics of DPP-IV substrates may be released upon enzymatic hydrolysis of milk proteins. Lacroix and Li-Chan (2012a) hypothesised that DPP-IV inhibition by various milk protein hydrolysates may actually involve milk protein-derived peptides behaving as DPP-IV substrates.

A link between the secondary complications of T2D such as cardiovascular disease

(atherosclerosis, stroke and coronary heart disease) and oxidative stress has been proposed. It has been suggested that natural antioxidants may be used as adjuncts to therapeutic approaches to help in preventing cardiovascular complications induced by T2D (Xu, Tappia, Neki & Dhalla, 2013). Milk proteins and peptides have been identified for their antioxidant properties. The utilization of anti-diabetic milk peptides/hydrolysates with additional antioxidant properties in the management of T2D has therefore been proposed (Nongonierma & FitzGerald, 2013).

Earlier studies have demonstrated the cleavage of milk protein-derived peptides by DPP-IV. However, to our knowledge, no studies have shown that milk protein-derived peptides display a prodrug- or substrate-type of inhibition towards DPP-IV. The aim of this study was to predict the release of DPP-IV substrate–like peptide sequences (peptides having a penultimate Pro residue) by gastrointestinal enzymes using an *in silico* digestion of the major individual milk proteins. Several casein-derived sequences were then synthesized and incubated with DPP-IV in order to assess their stability to further hydrolysis by DPP-IV. The parent peptides and their breakdown products were then tested for their DPP-IV inhibitory and antioxidant potential. A similar approach was followed with milk protein hydrolysates generated with gastrointestinal enzymes.

2. Materials and methods

2.1. Reagents

The synthetic peptides Leu-Pro, Tyr-Pro, Ile-Pro, Tyr-Tyr, Leu-Pro-Leu, Tyr-Pro-Tyr, Ile-Gln-Tyr, Tyr-Pro-Tyr-Tyr, Ile-Pro-Ile-Gln-Tyr, Leu-Pro-Leu-Pro-Leu and Leu-Pro-Tyr-Pro-Tyr were from Thermo Fisher Scientific (Ulm, Germany). Ile, Tyr, Leu, trifluoroacetic acid (TFA), tris(hydroxymethyl)aminomethane (TRIS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox^{TM}), Gly-Pro-pNA, diprotin A (Ile-Pro-Ile), ethanol, porcine DPP-IV ($\geq$ 10 units.mg^{-1} protein), high performance liquid chromatography (HPLC) grade water and acetonitrile (ACN) were obtained from Sigma Aldrich (Dublin, Ireland). Hydrochloric acid (HCl) and sodium hydroxide (NaOH) were from VWR (Dublin, Ireland).

2.2. In silico digestion of individual milk proteins with gastrointestinal enzymes and peptide

digestion with DPP-IV

In silico digestion of individual milk proteins was carried out with the peptide cutter program (ExPASy, 2011) using gastrointestinal enzymes (pepsin, trypsin and chymotrypsin). The different peptides released were then analysed for their potential to act as DPP-IV preferred substrates, i.e., peptides with a Pro residue at the penultimate position. Three casein-derived peptide sequences (Ile-Pro-Ile-Gln-Tyr, Leu-Pro-Leu-Pro-Leu and Leu-Pro-Tyr-Pro-Tyr) which contained previously identified DPP-IV inhibitory peptides within their sequence were selected from the *in silico* digestion with gastrointestinal enzymes. Ile-Pro-Ile-Gln-Tyr contained the potent DPP-IV inhibitor Ile-Pro-Ile which has also been identified as a DPP-IV substrate (Rahfeld et al., 1991). In addition, its incubation with DPP-IV was predicted to release Ile-Pro, which has been identified as a DPP-IV inhibitor (Hatanaka et al., 2012). Digestion of Leu-Pro-Leu-Pro-Leu and Leu-Pro-Tyr-Pro-Tyr with DPP-IV would theoretically release the previously identified DPP-IV inhibitors Leu-Pro and Tyr-Pro (Hatanaka et al., 2012). Tyr-Pro-Tyr-Tyr (Casoxin B) was used for its structural similarities with Leu-Pro-Tyr-Pro-Tyr. The five peptide sequences were digested *in silico* with DPP-IV and the resulting peptide products were identified.

2.3. Digestion of milk protein hydrolysates and peptides with DPP-IV

Peptides with a Pro residue at the penultimate position (position 2), including Tyr-Pro-Tyr, Leu-Pro-Leu, Leu-Pro-Leu-Pro-Leu, Tyr-Pro-Tyr-Tyr, Leu-Pro-Tyr-Pro-Tyr, Ile-Pro-Ile-Gln-Tyr and Ile-Pro-Ile, were subjected to DPP-IV digestion. The peptides Ile-Gln-Tyr and Pro-Ile, which did not present structural features of DPP-IV substrates, were used as negative controls. Peptides were resuspended in 100 mM Tris-HCl buffer pH 8.0 at 10 $mg.mL^{-1}$ and incubated at 37°C for 18 h with DPP-IV at two different enzyme to substrate ratios (E:S), 1 and 10 U DPP-IV: 1 g peptide. The control consisted of the peptide resuspended in 100 mM Tris-HCl buffer pH 8.0 at 10 $mg.mL^{-1}$ incubated at 37°C for 18 h without DPP-IV. The enzyme was heat inactivated at 90°C for 20 min, which resulted in complete inactivation of the enzyme as no DPP-IV activity was detected when the heat treated DPP-IV was subsequently incubated with Gly-Pro-pNA. In order

to check the retention of activity at the end of the reaction, DPP-IV was also incubated for 18 h at 37°C in 100 mM Tris-HCl buffer pH 8.0 without substrate. At the end of the incubation period, this sample was incubated with Gly-Pro-pNA and DPP-IV activity was determined.

Milk protein hydrolysates derived from caseins, whey proteins and lactoferrin as described in Nongonierma and FitzGerald (2013) were used in this study. The hydrolysates were resuspended in 100 mM Tris-HCl buffer pH 8.0 at 10 $mg.mL^{-1}$ (final concentration) and incubated at 37°C for 24 h with DPP-IV at an E:S of 0.2 and 2 U of DPP-IV.$g^{-1}$of hydrolysate. The control consisted of the hydrolysates resuspended in 100 mM Tris-HCl buffer pH 8.0 and incubated at 37°C for 24 h without DPP-IV. Hydrolysis with DPP-IV was carried out in duplicate (n=2).

2.4. DPP-IV inhibition assay

Diprotin A was resuspended in HPLC grade water at concentrations ranging from 12.5×10^{-3} to 12.5 $\mu g.mL^{-1}$ (final concentration), the other peptides and milk protein hydrolysates were dispersed at concentrations ranging from 12.5×10^{-3} to 1.25 $mg.mL^{-1}$ (final concentration). The DPP-IV inhibition assay was carried out as described by Nongonierma and FitzGerald (2013). Briefly, the test samples (25 μL) were pipetted onto a 96 well microplate (Sarstedt, Dublin, Ireland) containing Gly-Pro-pNA, the reaction substrate (50 μL, final concentration 0.2 mM). The negative control contained 100 mM Tris-HCl buffer pH 8.0 (25 μL) and the reaction substrate Gly-Pro-pNA. The reaction was initiated by the addition of DPP-IV (50 μL, final concentration 0.0025 $U.mL^{-1}$). All the reagents and samples were diluted in 100 mM Tris-HCl buffer pH 8.0. Diprotin A was used as a positive control. Each sample was analysed in triplicate. The microplate was incubated at 37°C for 60 min in a microplate reader (Biotek Synergy HT, Winoosky, VT, USA), absorbance of the released pNA was monitored at 405 nm. The DPP-IV IC_{50} values (concentration of active compound required to observe 50 % DPP-IV inhibition) were determined by plotting the percentage inhibition as a function of the concentration of test compound.

Lineweaver and Burk analysis was used to study the mode of inhibition as described by Nongonierma and FitzGerald (2013). The initial rate of the reaction (pNA released from Gly-Pro-pNA) was measured at different Gly-Pro-pNA concentrations ranging between 0.2 to 0.6 mM in the presence and absence of the DPP-IV peptide inhibitors at their IC_{50} concentration. The affinity constant (Km, determined without inhibitor), apparent affinity constant (Kapp, determined in the presence of DPP-IV inhibitor) and the maximum rate of the reaction (Vmax) were determined from the double reciprocal plots.

2.5. DPPH radical scavenging assay

The DPPH assay was used to determine the radical scavenging properties of the peptides which were dispersed in HPLC grade water at concentrations ranging from 1.25×10^{-2} to 2.5 $mg.mL^{-1}$. The DPPH scavenging assay was carried out essentially according to Nongonierma and FitzGerald (2013). Briefly, the test samples (50 µL) were pipetted onto a 96 well microplate containing 150 µL of a DPPH (final concentration 0.088 mM) solution in 50 % (v/v) ethanol. The microplate was incubated at 37°C for 60 min in a microplate reader, absorbance of the DPPH radical was monitored at 517 nm. Each sample was analysed in triplicate. Trolox was used as a positive control. Scavenging of the DPPH radical was determined with respect to a control containing no scavenger (DPPH solution added with 50 µL water). The DPPH scavenging EC_{50} values (concentration of active compound required to observe 50 % DPPH scavenging) were determined by plotting the percentage DPPH scavenging as a function of the concentration of test compound.

2.6. Reverse-phase ultra-performance liquid chromatography (RP-UPLC) of peptides and hydrolysates

Profiles of different samples, including peptides and milk protein hydrolysates, before and after incubation with DPP-IV at 37°C were determined by reverse-phase (RP) using an ultra-performance liquid chromatograph (UPLC Acquity - Waters, Dublin, Ireland) equipped with a 2.1 x 50 mm, 1.7 µm Acquity UPLC C18 BEH column mounted with a 0.2 µm inline filter

(Waters) as described by Nongonierma and FitzGerald (2012). All peptides, including the DPP-IV substrate-like peptides and their predicted breakdown products were injected in triplicate (n=3) onto the UPLC column at different concentrations (25, 50, 100, 150, 200 and 250 $\mu g.mL^{-1}$). Retention time of the standards was used to identify amino acids and peptides present in the samples obtained before and after incubation with DPP-IV. The calibration curves (peak area as a function of peptide concentration, $R^2 > 0.95$) for all peptides were generated and used to quantify the amount of each amino acid and peptide present in the samples before and after incubation with DPP-IV.

2.7. Statistical analysis

Means comparison was carried out using a one way ANOVA followed by a Student Newman-Keuls test using SPSS (version 9, SPSS Inc., Chicago, IL, USA) at a significance level $P < 0.05$.

3. Results

3.1. In silico gastrointestinal digestion of DPP-IV inhibitory peptides

Of the different milk protein-derived peptides identified with DPP-IV preferred substrate features, three peptide sequences Ile-Pro-Ile-Gln-Tyr (κ-casein variant A, f26-30), Leu-Pro-Tyr-Pro-Tyr (κ-casein variant A, f56-60) and Leu-Pro-Leu-Pro-Leu (β-casein variant A2, f135-139) were chosen for this study. These were selected on the basis that they contained previously identified DPP-IV inhibitory peptides which may be released during DPP-IV digestion. In addition, Tyr-Pro-Tyr-Tyr (κ-casein variant A, f58-61) which has previously been identified as casoxin B (Chiba, Tani & Yoshikawa, 1989) and diprotin A (Ile-Pro-Ile, κ-casein variant A, f26-28) were also included in this study. Ile-Pro-Ile, a well-known DPP-IV inhibitor, was used as a positive control as it has previously been identified as a DPP-IV substrate (Rahfeld et al., 1991).

In silico digestion of these peptide sequences by DPP-IV allowed prediction of the potential breakdown products. The possible DPP-IV cleavage sites on the peptides are illustrated in Fig. 1. This analysis predicted that digestion of Ile-Pro-Ile-Gln-Tyr with DPP-IV would yield Ile-Pro and Ile-Gln-Tyr; Leu-Pro-Leu-Pro-Leu was predicted to yield Leu-Pro, Leu-Pro-Leu, and Leu; Leu-

Pro-Tyr-Pro-Tyr was predicted to yield Leu-Pro, Tyr-Pro-Tyr, Tyr-Pro and Tyr; Tyr-Pro-Tyr-Tyr was predicted to yield Tyr-Pro and Tyr-Tyr while Ile-Pro-Ile was predicted to yield Ile-Pro and Ile. The five parent peptides and their predicted breakdown products were investigated for their DPP-IV inhibitory properties and their further susceptibility to cleavage by DPP-IV *in vitro*.

3.2. DPP-IV inhibition and antioxidant activity of the milk protein-derived peptides

Of the twelve peptides tested, ten (Leu-Pro, Tyr-Pro, Ile-Pro, Ile-Pro-Ile, Leu-Pro-Leu, Tyr-Pro-Tyr, Tyr-Pro-Tyr-Tyr, Ile-Pro-Ile-Gln-Tyr, Leu-Pro-Leu-Pro-Leu and Leu-Pro-Tyr-Pro-Tyr) were able to inhibit DPP-IV and two peptides were found to be inactive (Tyr-Tyr and Ile-Gln-Tyr). The IC_{50} for the ten DPP-IV inhibitory peptides identified was determined (Table 1). The lowest IC_{50} value was observed for Ile-Pro-Ile (3.4 ± 0.1 μM), which was ten times lower than that of Ile-Pro-Ile-Gln-Tyr (35.2 ± 1.8 μM). The least potent compound studied herein was Leu-Pro (712.5 ± 11.0 μM), which was ~ 200 times less potent than Ile-Pro-Ile.

The mode of DPP-IV inhibition for the peptides was determined using the Lineweaver and Burk representation. The Lineweaver and Burk double reciprocal plots for Ile-Pro-Ile, Ile-Pro-Ile-Gln-Tyr, Leu-Pro-Leu-Pro-Leu, Leu-Pro-Tyr-Pro-Tyr, Tyr-Pro-Tyr and Leu-Pro are illustrated in supplementary Fig. S1. For all peptides studied, there was no significant difference for Vmax determined with or without inhibitor ($P \geq 0.05$), whereas Km was significantly different ($P < 0.05$) from Kapp. These results suggested that all DPP-IV inhibitory peptides studied herein were competitive inhibitors of DPP-IV and therefore could directly bind to its active site.

The antioxidant activity of the twelve peptides was evaluated by determining their ability to scavenge the DPPH radical. Ile-Pro-Ile-Gln-Tyr, Leu-Pro-Tyr-Pro-Tyr, Tyr-Pro-Tyr-Tyr, Tyr-Pro-Tyr and Tyr-Tyr were able to scavenge DPPH (Table 1). The other peptides did not show any scavenging activity toward DPPH radicals. The antioxidant potency of the peptides was evaluated by determining their EC_{50} value. The EC_{50} values were relatively high suggesting that these peptides did not have good antioxidant properties at least *in vitro*.

3.3. Degradation of milk protein-derived peptides and milk protein hydrolysates following

246 *incubation with DPP-IV*

247 Peptides with DPP-IV preferred substrate features (Ile-Pro-Ile, Leu-Pro-Tyr-Pro-Tyr, Leu-Pro-
248 Leu-Pro-Leu, Ile-Pro-Ile-Gln-Tyr, Tyr-Pro-Tyr-Tyr, Leu-Pro-Leu and Tyr-Pro-Tyr) were
249 incubated with DPP-IV at a low and high E:S. After 18 h incubation at 37°C with DPP-IV, all
250 peptides were cleaved by the enzyme with the exception of Ile-Gln-Tyr and Pro-Ile (the negative
251 controls) which in theory could not be cleaved by DPP-IV. It was found at the end of the
252 incubation that DPP-IV was still active as 69.3 ± 2.9 % DPP-IV activity was found. The RP-
253 UPLC profiles for Ile-Pro-Ile, Ile-Pro-Ile-Gln-Tyr, Leu-Pro-Leu-Pro-Leu and Leu-Pro-Tyr-Pro-
254 Tyr incubated at the low and high E:S are illustrated on Fig. 2. The RP-UPLC profiles show that
255 these peptides were cleaved to release their predicted breakdown products as per *in silico*
256 digestion with DPP-IV (section 3.1.). As expected peptide breakdown was more pronounced at
257 the high compared to the low E:S. The same results were found with Tyr-Pro-Tyr-Tyr, Tyr-Pro-
258 Tyr and Leu-Pro-Leu (data not shown).

259 Quantification of the peptides in each sample was carried out using the response factors from
260 the calibration curves determined for each individual peptide and amino-acid. The concentration
261 of amino acids and peptides present in each sample is shown in Table 2. As expected, breakdown
262 of the parent peptide was higher at the high compared to the low E:S. The mass balance of
263 peptides generated during DPP-IV hydrolysis agreed with the concentration of parent peptide
264 cleaved. Some components were not detected in the hydrolysed samples, this was due to the
265 amount of amino acids or peptides being below the detection threshold. Differences were seen in
266 the extent of peptide breakdown depending on the sequence of the parent peptide. At the high
267 E:S, Tyr-Pro-Tyr-Tyr and Tyr-Pro-Tyr were completely cleaved and could not be detected after
268 18 h incubation. In contrast, modest peptide breakdown was seen with Ile-Pro-Ile with less than
269 10 % (w/w) of this peptide being cleaved at high E:S.

270 Three milk protein hydrolysates studied herein which have previously been shown to be DPP-
271 IV inhibitors (Nongonierma & FitzGerald, 2013) were incubated with DPP-IV. The RP-UPLC

profiles for the high E:S digests are illustrated on supplementary Fig. S2. Slight differences were seen on the RP-UPLC profiles of the casein hydrolysate (CasH) before and after incubation with DPP-IV at 2, 20, 21 and 28 min retention times, where peptide peaks did not match between the control and the hydrolysate incubated with DPP-IV (supplementary Fig. S2 a). For the whey protein hydrolysate (WPH), one peak eluting at 15 min was absent from the hydrolysate after incubation with DPP-IV (supplementary Fig. S2 b). In the case of the lactoferrin hydrolysate (LFH), slight differences were seen at 3 and 9 min between the control and after incubation with DPP-IV (supplementary Fig. S2 c).

Overall, incubation of the individual peptides with DPP-IV induced major changes in the peptide composition, whereas for the milk protein hydrolysate, only minor modifications were seen.

3.4. DPP-IV inhibitory properties of milk protein-derived peptides and milk protein hydrolysates following incubation with DPP-IV

The IC_{50} values for the peptides pre and post DPP-IV hydrolysis was evaluated to study the impact of peptide breakdown on the bioactive properties (Table 3). The IC_{50} value of the control samples were similar to the values previously reported for these peptides (Table 1), suggesting that the bioactive properties of these peptides were not altered following incubation at 37°C for 18 h. In addition, the RP-UPLC of the peptides in the control and freshly prepared peptides did not differ (data not shown), suggesting that the peptides were not degraded during incubation in the controls. For most peptides, there was no significant difference ($P \geq 0.05$) in terms of IC_{50} value between the control and the low E:S sample, with the exception of Leu-Pro-Leu-Pro-Leu. Leu-Pro-Leu-Pro-Leu had a significantly ($P < 0.05$) lower IC_{50} value at the low E:S compared to the control. For all peptides, incubation with DPP-IV at the high E:S resulted in a significant increase ($P < 0.05$) in the IC_{50} value compared to the control and the low E:S sample. However, this was not seen for Leu-Pro-Leu-Pro-Leu where the IC_{50} value significantly decreased ($P < 0.05$) following incubation with DPP-IV and no significant difference ($P \geq 0.05$) was seen

between the IC_{50} values at the low and high E:S.

The IC_{50} values for the milk protein hydrolysates incubated with DPP-IV at a high and low E:S were determined (Fig. 3). There was no significant difference ($P \geq 0.05$) between the IC_{50} value of the three hydrolysate controls. Following incubation with DPP-IV, no significant differences in terms of IC_{50} values were found for WPH and LFH between the control and the high and low E:S digests. In contrast, for CasH, samples incubated with DPP-IV displayed a significantly lower ($P < 0.05$) IC_{50} value compared to CasH control. There was no significant difference ($P \geq 0.05$) between CasH incubated at high or low E:S.

4. Discussion

This study has demonstrated that milk protein-derived peptides, with a Pro at position 2, predicted to be released by gastrointestinal enzymes could act as DPP-IV inhibitors, involving a substrate or prodrug mode of inhibition. With both substrate or prodrug modes of inhibition, the starting peptides are susceptible to further DPP-IV cleavage, resulting in the release of more or less potent peptide inhibitors as compared to the parent peptide. All peptides studied herein were competitive inhibitors, suggesting a direct interaction of the peptides with the active site of DPP-IV.

To our knowledge, of the different peptides studied, only Ile-Pro-Ile, Ile-Pro, Tyr-Pro and Leu-Pro have previously been identified for their DPP-IV inhibitory properties (Hatanaka et al., 2012). In agreement with the results of Hatanaka et al. (2012), Ile-Pro also displayed a lower IC_{50} value compared to Leu-Pro (149.6 ± 6.1 and 712.5 ± 11.0 μM, respectively, Table 1). In addition, Ile-Pro was about 6 times more potent than Leu-Pro, which is of the same order as the values reported in Table 1. In contrast, we found that Tyr-Pro was a more potent DPP-IV inhibitor than Leu-Pro, whereas Hatanaka et al. (2012) reported the opposite trend.

Relatively potent DPP-IV inhibitors displaying a substrate-type inhibition have been reported in the literature. The well-known inhibitors diprotin A (Ile-Pro-Ile) and diprotin B (Val-Pro-Leu) isolated from microbial cultures have previously been shown to behave as DPP-IV substrates

(Rahfeld et al., 1991). Other DPP-IV inhibitory peptides isolated from casein with a Pro at the penultimate position, have recently been identified, these include Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu, Leu-Pro-Gln and Val-Pro-Ile-Thr-Pro-Thr-Leu with IC_{50} values of 46, 82 and 110 µM, respectively (Uenishi et al., 2012). Similarly, Ile-Pro-Ala (Tulipano et al., 2011) and Ile-Pro-Ala-Val-Phe (Silveira et al., 2013) were isolated from β-lactoglobulin, with DPP-IV IC_{50} values of 49 and 45 µM, respectively. Results obtained from DPP-IV inhibitory potency correlated with the composition of the samples, with the overall IC_{50} value being governed by the amount of the most potent peptide within the sample (Table 2 and 3). Similar results have previously been reported by Hatanaka et al. (2012) with Diprotin A (Ile-Pro-Ile) having an IC_{50} value twice as low as its breakdown product Ile-Pro (0.21 ± 0.01 mM and 0.41 ± 0.07 mM, respectively). For illustration, in the case of Tyr-Pro-Tyr and Tyr-Pro-Tyr-Tyr incubated at a high DPP-IV E:S, all the parent peptide was degraded. As a consequence, these samples only contained Tyr-Pro as DPP-IV inhibitory peptide. Interestingly, the IC_{50} value for Tyr-Pro-Tyr and Tyr-Pro-Tyr-Tyr incubated at high E:S of DPP-IV (523.4 ± 31.9 and 783.7 ± 50.6 µM, respectively, Table 3) was of the same order as that of Tyr-Pro (658.1 ± 8.0 µM, Table 1).

To our knowledge, no study has identified milk protein-derived peptides with a prodrug-type behavior for DPP-IV inhibition. One peptide, Leu-Pro-Leu-Pro-Leu, had a different behaviour from the other peptides studied herein, showing a decrease in the IC_{50} value following incubation at low and high E:S as compared to the control (Table 3). This peptide therefore displayed a prodrug-type inhibition according to the classification established by Fujita & Yoshikawa (1999). In the sample containing Leu-Pro-Leu-Pro-Leu incubated with low and high E:S, significant amounts of Leu-Pro-Leu were found (Table 2). This peptide is about 30 % more potent than Leu-Pro-Leu-Pro-Leu and three times more potent than Leu-Pro, which explains the overall decrease in IC_{50} value seen following incubation of Leu-Pro-Leu-Pro-Leu with DPP-IV.

Relatively high amounts of Leu-Pro-Leu and Tyr-Pro-Tyr, which could be substrates for DPP-IV were also found with the parent peptides Leu-Pro-Leu-Pro-Leu and Tyr-Pro-Tyr-Pro-Tyr,

respectively, incubated with DPP-IV at high E:S. The susceptibility of Leu-Pro-Leu and Tyr-Pro-Tyr to DPP-IV cleavage varied when these sequences were encrypted in a larger peptide (Leu-Pro-Leu-Pro-Leu and Leu-Pro-Tyr-Pro-Tyr, respectively). At the high E:S, around 35 % of Tyr-Pro-Tyr was cleaved from Leu-Pro-Tyr-Pro-Tyr, whereas 100 % of Tyr-Pro-Tyr was cleaved with DPP-IV. For Leu-Pro-Leu incubated with DPP-IV, more than 75 % was cleaved whereas 30 % Leu-Pro-Leu was cleaved when Leu-Pro-Leu-Pro-Leu was incubated with DPP-IV at the high E:S. These differences may arise from the inhibition exerted by the parent peptide (Leu-Pro-Leu-Pro-Leu or Leu-Pro-Tyr-Pro-Tyr) on DPP-IV, which may have blocked further cleavage of Tyr-Pro-Tyr and Leu-Pro-Leu. This is further supported by the fact that the more potent DPP-IV inhibitory peptides (i.e. Ile-Pro-Ile, Ile-Pro-Ile-Gln-Tyr and Leu-Pro-Tyr-Pro-Tyr, Table 1) studied herein were generally cleaved to a lesser extent by DPP-IV (Table 2 and Fig. 2).

For the whey protein-derived hydrolysates (WPH and LFH), preincubation with DPP-IV did not affect the DPP-IV inhibitory properties of the samples (Fig. 3). This was in agreement with the fact that the peptide profile for these samples was essentially unaffected by incubation with DPP-IV (supplementary Fig. S2). However, with CasH, more obvious modifications were seen in the peptide profile, which resulted in a significant decrease in the DPP-IV inhibitory properties. Differences observed between whey proteins and caseins may come from the fact that caseins contain higher amounts of Pro residues on a weight basis compared to whey. In addition, a higher number of peptides with a Pro at the penultimate position were identified with the *in silico* approach used herein for the caseins (α_{s1}-, α_{s2}-, β- and κ-casein) as compared to the whey proteins (β-lactoglobulin and α-lactalbumin). The significant decrease in the IC_{50} value observed with CasH following incubation with DPP-IV may be explained by the fact that it may contain prodrug-type DPP-IV inhibitory peptides. Peptide peaks eluting at the same retention time as Leu-Pro-Leu-Pro-Leu (17.9 min) and Leu-Pro-Leu (12.3 min) were seen on the RP-UPLC profile of CasH. However, there was no difference in peak areas between the control and the high E:S digest of CasH, suggesting that these peptides may not be responsible for the decreased IC_{50}

value. However, several other peptides which have not been studied herein may be responsible for this effect. Mass spectrometric identification of the peptides within CasH may help to better understand the effect of DPP-IV pre-digestion on the overall DPP-IV inhibitory properties of the casein hydrolysate.

To assess if the peptides studied herein also had the potential to reduce oxidative stress in T2D, their antioxidant activity was studied. The peptides evaluated herein only had a modest DPPH scavenging activity. The EC_{50} value for DPPH scavenging by the casein-derived peptide Tyr-Pro-Tyr-Pro-Glu-Leu was 98 μM while it was 23.3 μM for carnosine (Ala-His) (Suetsuna, Ukeda & Ochi, 2000). EC_{50} values of 242 and 654 μM have been reported for Trp-Val and Val-Trp, respectively (Nongonierma & FitzGerald, 2013).

A wide range of biologically active peptides, including immunomodulatory, neuro- and vaso-active peptides, may be cleaved *in vivo* by DPP-IV resulting, in some cases, in the alteration of their biological activity (Vanhoof et al., 1995). In agreement with our results, relatively long incubation times with DPP-IV were required to achieve significant cleavage of the substrate. For instance, with [(Xaa-Pro)n]−[drug] conjugates, it has been shown that up to 92 % of the conjugates were cleaved by DPP-IV following a 24 h incubation period (García-Aparicio et al., 2006). Cleavage of the milk protein-derived peptide, β-casomorphin (f1-5) (Tyr-Pro-Phe-Pro-Gly), by DPP-IV from renal brush border membrane has been demonstrated (Miyamoto, Ganapathy, Barlas, Neubert, Barth & Leibach, 1987; Tiruppathi, Miyamoto, Ganapathy, Roesel, Whitford & Leibach, 1990). In addition, it was also shown that cleavage of Pro containing peptides and gliadin, a Pro rich protein, also occurred with DPP-IV from intestinal brush border membrane of rat (Tiruppathi, Miyamoto, Ganapathy & Leibach, 1993). It was also demonstrated that Leu-Pro-Gly-Gly was degraded in Leu-Pro and Gly-Gly by DPP-IV located in the intestinal brush border membrane of rat (Morita, Chung, Freeman, Erickson, Sleisenger & Kim, 1983). Based on the available scientific evidence, it is anticipated that the results obtained *in vitro* with the milk protein-derived peptides studied herein may also translate *in vivo*. The different milk

protein-derived peptides studied herein may be further degraded by DPP-IV in the gastrointestinal tract to release amino acids and smaller peptides as predicted by the *in silico* digestion with DPP-IV (Fig. 1).

Degradation of Pro containing peptides by DPP-IV located in the intestinal brush border membrane *in vivo* has been shown to directly affect absorption of peptides. Morita et al. (1983) have shown that ileal absorption rate of the constitutive amino acids of Leu-Pro-Gly-Gly (i.e. Leu, Pro and Gly) and Leu-Pro and Gly-Gly was faster than that of the tetrapeptide. Relatively high levels of DPP-IV (1.03 $g.mL^{-1}$) have been identified in the serum of humans (Cuchacovich, Gatica, Pizzo & Gonzalez-Gronow, 2001). If they can cross the gut barrier, it is anticipated that the short peptides studied herein (Leu-Pro-Leu and Tyr-Pro-Tyr) may be degraded into Leu-Pro and Tyr-Pro. Leu-Pro and Tyr-Pro could in turn inhibit DPP-IV in the circulation. The peptide sequences studied herein are of relevance to multi-site targeting for DPP-IV inhibition. They may display their DPP-IV inhibitory potential directly in the gastrointestinal tract where they may be degraded by intestinal brush border DPP-IV (Tiruppathi et al., 1993). This in turn will release shorter peptides which are better candidates for intestinal permeation possibly allowing these to reach the circulation where they may further display their DPP-IV inhibitory properties. Although the IC_{50} value of the larger peptides was generally found to be lower than that of the shorter peptides studied herein, the IC_{50} values for shorter peptides indicate that these are still moderately potent inhibitors of DPP-IV (Table 1).

Conclusion

Milk protein-derived peptides which have been predicted to be released by gastrointestinal enzyme activities have been shown to behave as substrate- or prodrug-type inhibitors of DPP-IV. Besides their DPP-IV inhibitory properties, which make them interesting candidates for protecting incretins against DPP-IV cleavage in the gastrointestinal tract, the instability of these peptides may be further exploited. Cleavage of these peptide sequences by DPP-IV was shown to

release peptides which were in some cases more or less potent DPP-IV inhibitors. It is anticipated that such peptides, owing to their small size and relatively high hydrophobicity may be good candidates for intestinal absorption, thereby, allowing them to reach the circulation where they may display their bioactive properties. Validation of these results *in vivo* is still required, however, based on numerous studies conducted *in vivo* with peptides showing DPP-IV substrate-like features, it is anticipated that similar trends may be found *in vivo*. To our understanding, the results presented herein are relevant to the management of type 2 diabetes with functional foods involving multi-target sites for DPP-IV inhibition in humans.

Acknowledgements

The work described herein was supported by Enterprise Ireland under Grant Number CC20080001. The authors wish to acknowledge Ms. Niamh Davern for her technical assistance.

441 **References**

442 Bjelke, J. R., Christensen, J., Nielsen, P. F., Branner, S., Kanstrup, A. B., Wagtmann, N., &
443 Rasmussen, H. B. (2006). Dipeptidyl peptidases 8 and 9: specificity and molecular
444 characterization compared with dipeptidyl peptidase IV. *Biochemistry Journal, 396*, 391-
445 399.

446 Chiba, H., Tani, F., & Yoshikawa, M. (1989). Opioid antagonist peptides derived from β-casein.
447 *Journal of Dairy Research, 56*, 363-366.

448 Cuchacovich, M., Gatica, H., Pizzo, S., & Gonzalez-Gronow, M. (2001). Characterization of
449 human serum dipeptidyl peptidase IV (CD26) and analysis of its autoantibodies in
450 patients with rheumatoid arthritis and other autoimmune diseases. *Clinical and*
451 *experimental rheumatology, 19*, 673-680.

452 ExPASy. (2011). Swiss Institute of Bioinformatics, bioinformatics resource portal. URL:
453 http://web.expasy.org/peptide_cutter/. Acessed on the 28th March 2014.

454 Fujita, H., & Yoshikawa, M. (1999). LKPNM: a prodrug-type ACE-inhibitory peptide derived
455 from fish protein. *Immunopharmacology, 44*, 123-127.

456 García-Aparicio, C., Bonache, M.-C., De Meester, I., San-Félix, A., Balzarini, J., Camarasa, M.-
457 J., & Velázquez, S. (2006). Design and discovery of a novel dipeptidyl-peptidase IV
458 (CD26)-based prodrug approach. *Journal of Medicinal Chemistry, 49*, 5339-5351.

459 Hatanaka, T., Inoue, Y., Arima, J., Kumagai, Y., Usuki, H., Kawakami, K., Kimura, M., &
460 Mukaihara, T. (2012). Production of dipeptidyl peptidase IV inhibitory peptides from
461 defatted rice bran. *Food Chemistry, 134*, 797-802.

462 Huang, S.-L., Jao, C.-L., Ho, K.-P., & Hsu, K.-C. (2012). Dipeptidyl-peptidase IV inhibitory

activity of peptides derived from tuna cooking juice hydrolysates. *Peptides, 35*, 114-121.

Kühn-Wache, K., Bär, J. W., Hoffmann, T., Wolf, R., Rahfeld, J.-U., & Demuth, H.-U. (2011). Selective inhibition of dipeptidyl peptidase 4 by targeting a substrate-specific secondary binding site. *Biological Chemistry, 392*, 223-231.

Lacroix, I. M., & Li-Chan, E. C. Y. (2013). Inhibition of dipeptidyl peptidase (DPP)-IV and α-glucosidase activities by pepsin-treated whey proteins. *Journal of Agricultural and Food Chemistry, 61*, 7500–7506.

Lacroix, I. M. E., & Li-Chan, E. C. Y. (2012a). Dipeptidyl peptidase-IV inhibitory activity of dairy protein hydrolysates. *International Dairy Journal, 25*, 97-102.

Lacroix, I. M. E., & Li-Chan, E. C. Y. (2012b). Evaluation of the potential of dietary proteins as precursors of dipeptidyl peptidase (DPP)-IV inhibitors by an *in silico* approach. *Journal of Functional Foods, 4*, 403-422.

Miyamoto, Y., Ganapathy, V., Barlas, A., Neubert, K., Barth, A., & Leibach, F. H. (1987). Role of dipeptidyl peptidase IV in uptake of peptide nitrogen from beta-casomorphin in rabbit renal BBMV. *American Journal of Physiology - Renal Physiology, 252*, F670-F677.

Morita, A., Chung, Y., Freeman, H., Erickson, R., Sleisenger, M., & Kim, Y. (1983). Intestinal assimilation of a proline-containing tetrapeptide. Role of a brush border membrane postproline dipeptidyl aminopeptidase IV. *Journal of Clinical Investigation, 72*, 610.

Nongonierma, A. B., & FitzGerald, R. J. (2012). Tryptophan-containing milk protein-derived dipeptides inhibit xanthine oxidase. *Peptides, 37*, 263-272.

Nongonierma, A. B., & FitzGerald, R. J. (2013). Dipeptidyl peptidase IV inhibitory and antioxidative properties of milk-derived dipeptides and hydrolysates. *Peptides, 39*, 157-

163.

Rahfeld, J., Schierborn, M., Hartrodt, B., Neubert, K., & Heins, J. (1991). Are diprotin A (Ile-Pro-Ile) and diprotin B (Val-Pro-Leu) inhibitors or substrates of dipeptidyl peptidase IV? *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology, 1076*, 314-316.

Silveira, S. T., Martínez-Maqueda, D., Recio, I., & Hernández-Ledesma, B. (2013). Dipeptidyl peptidase-IV inhibitory peptides generated by tryptic hydrolysis of a whey protein concentrate rich in β-lactoglobulin. *Food Chemistry, 141*, 1072–1077.

Suetsuna, K., Ukeda, H., & Ochi, H. (2000). Isolation and characterization of free radical scavenging activities peptides derived from casein. *The Journal of Nutritional Biochemistry, 11*, 128-131.

Tiruppathi, C., Miyamoto, Y., Ganapathy, V., & Leibach, F. H. (1993). Genetic evidence for role of DPP IV in intestinal hydrolysis and assimilation of prolyl peptides. *American Journal of Physiology - Gastrointestinal and Liver Physiology, 265*, G81-G89.

Tiruppathi, C., Miyamoto, Y., Ganapathy, V., Roesel, R. A., Whitford, G. M., & Leibach, F. H. (1990). Hydrolysis and transport of proline-containing peptides in renal brush-border membrane vesicles from dipeptidyl peptidase IV-positive and dipeptidyl peptidase IV-negative rat strains. *Journal of Biological Chemistry, 265*, 1476-1483.

Tulipano, G., Sibilia, V., Caroli, A. M., & Cocchi, D. (2011). Whey proteins as source of dipeptidyl dipeptidase IV (dipeptidyl peptidase-4) inhibitors. *Peptides, 32*, 835-838.

Uchida, M., Ohshiba, Y., & Mogami, O. (2011). Novel dipeptidyl peptidase-4-inhibiting peptide derived from β-lactoglobulin. *Journal of Pharmacological Sciences, 117*, 63-66.

Uenishi, H., Kabuki, T., Seto, Y., Serizawa, A., & Nakajima, H. (2012). Isolation and identification of casein-derived dipeptidyl-peptidase 4 (DPP-4)-inhibitory peptide LPQNIPPL from gouda-type cheese and its effect on plasma glucose in rats. *International Dairy Journal, 22*, 24-30.

Vanhoof, G., Goossens, F., De Meester, I., Hendriks, D., & Scharpé, S. (1995). Proline motifs in peptides and their biological processing. *The Federation of American Societies for Experimental Biology Journal, 9*, 736-744.

Xu, Y.-J., Tappia, P. S., Neki, N. S., & Dhalla, N. S. (2013). Prevention of diabetes-induced cardiovascular complications upon treatment with antioxidants. *Heart failure reviews*, 1-9.

Table 1 Peptide concentration inducing 50 % inhibition (IC_{50}) for dipeptidyl peptidase IV (DPP-IV) and concentration of peptide required to observe 50 % 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging (EC_{50}) in the presence of milk protein-derived peptides.

Peptide sequence	**DPP IV IC_{50} (µM)***	**DPPH EC_{50} (mM)***
IPI (diprotin A)	3.4 ± 0.1^{a}	nd
IPIQY	35.2 ± 1.8^{b}	4.18 ± 0.61
LPYPY	108.3 ± 2.8^{c}	>5
IP	149.6 ± 6.1^{d}	-
YPYY	194.4 ± 13.0^{e}	> 10
LPL	241.4 ± 11.4^{f}	-
YPY	243.7 ± 2.8^{f}	-
LPLPL	325.0 ± 15.2^{g}	-
YP	658.1 ± 8.0^{h}	>5
LP	712.5 ± 11.0^{h}	-
YY	-	>5
IQY	-	-
Trolox ($\times 10^3$)	nd	17.2 ± 5.5

*Values represent mean IC_{50} values ± confidence interval ($P = 0.05$), n=3. Values with different superscript letters are significantly different ($P < 0.05$).
nd: not determined, -: no activity detected.

Table 2 Concentration of milk protein-derived peptides pre- (control) and post-hydrolysis with dipeptidyl peptidase IV (DPP-IV) following incubation at 37°C for 18 h at a low (1 U: 1 g peptide) and high (10 U: 1 g peptide) enzyme to substrate ratio (E: S).

Parent peptide	Predicted sequences post DPP-IV hydrolysis	Peptide or amino acid concentration (μM)		
		Control	Low E:S	High E:S
IPI (diprotin A)	IPI	0.868 ± 0.001	0.854 ±0.002	0.801 ± 0.047
	IP	nd	nd	0.072 ± 0.002
	I	nd	0.020±0.017	0.076 ± 0.043
LPYPY	LPYPY	0.408 ± 0.001	0.360 ± 0.001	0.172 ± 0.021
	LP	nd	0.036 ± 0.001	0.182 ± 0.064
	YPY	nd	0.034 ± 0.001	0.192 ± 0.001
	YP	nd	nd	0.042 ± 0.001
	Y	nd	nd	0.052 ± 0.001
LPLPL	LPLPL	0.468 ± 0.012	0.288 ± 0.001	0.099 ± 0.001
	LPL	nd	0.156 ± 0.002	0.256 ± 0.001
	LP	nd	0.100 ± 0.001	0.487 ± 0.003
	L	nd	nd	nd
IPIQY	IPIQY	0.450 ± 0.001	0.450 ± 0.001	0.264 ± 0.001
	IP	nd	0.026 ± 0.001	0.158 ± 0.001
	IQY	nd	0.023 ± 0.001	0.162 ± 0.001
YPYY	YPYY	0.441 ± 0.001	0.420 ± 0.001	nd
	YP	nd	0.074 ± 0.001	0.385 ± 0.001
	YY	nd	0.078 ± 0.001	0.397 ± 0.001
LPL	LPL	0.817 ± 0.004	0.801 ± 0.003	0.106 ± 0.055
	LP	nd	0.076 ± 0.002	0.471 ± 0.002
	L	nd	nd	nd
YPY	YPY	0.501 ± 0.001	0.446 ± 0.047	nd
	YP	nd	0.037 ± 0.024	0.445 ± 0.001
	Y	nd	0.048 ± 0.001	0.430 ± 0.001
PI	PI	1.171 ± 0.004	1.208 ± 0.002	1.239 ± 0.001
IQY	IQY	0.506 ± 0.001	0.469 ± 0.001	0.492 ± 0.001

nd: not detected

Table 3 Concentration of milk protein-derived peptides inducing 50 % inhibition (IC_{50}) of dipeptidyl peptidase IV (DPP-IV) pre- (control) and post-hydrolysis of the milk protein-derived peptides with DPP-IV following incubation at 37°C for 18 h at a low (1 U: 1 g peptide) and high (10 U: 1 g peptide) enzyme to substrate ratio (E: S).

Compound	**DPP-IV IC_{50}***		
	Control	**Low E:S**	**High E:S**
IPI (diprotin A)	2.9 ± 0.2^{a}	3.3 ± 0.1^{a}	4.4 ± 0.3^{b}
IPIQY	26.7 ± 0.6^{c}	31.1 ± 0.3^{c}	40.4 ± 0.4^{d}
LPYPY	90.8 ± 2.8^{e}	89.7 ± 7.7^{e}	124.1 ± 23.3^{f}
LPL	186.8 ± 3.1^{g}	$195.8 \pm 3.5^{g,h}$	542.2 ± 9.1^{k}
YPYY	$207.9 \pm 9.2^{g,h}$	$238.3 \pm 48.1^{h,i}$	523.4 ± 31.9^{k}
YPY	282.0 ± 26.8^{j}	387.0 ± 40.2^{j}	783.7 ± 50.6^{l}
LPLPL	358.4 ± 15.6^{j}	271.6 ± 18.6^{i}	$246.7 \pm 66.5^{h,i}$

*Values represent mean IC_{50} values ± confidence interval ($P = 0.05$), n=3. Values with different superscript letters are significantly different ($P < 0.05$).